Nitrogen-Containing Phorbol Esters from *Croton ciliatoglandulifer* and Their Effects on Cyclooxygenases-1 and -2

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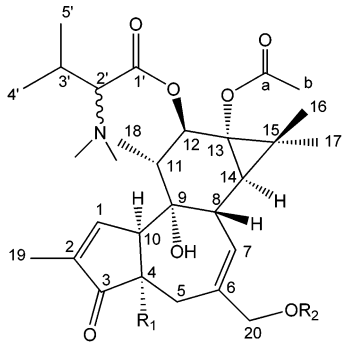
Four new phorbol derivatives, 12-*O*-[(2*R*)-*N*,*N*-dimethyl-3-methylbutanoyl]-4-deoxyphorbol 13-acetate (**1**), 12-*O*-[(2*S*)-*N*,*N*-dimethyl-3-methylbutanoyl]-4-deoxyphorbol 13-acetate (**2**), 12-*O*-[3-methyl-2-butenoyl]-4-deoxyphorbol 13-acetate (**3**), and 12-*O*-[(2*R*)-*N*,*N*-dimethyl-3-methylbutanoyl]phorbol 13-acetate (**4**), along with six known compounds, were isolated from the aerial parts of *Croton ciliatoglandulifer*. An anti-inflammatory activity of a hexane extract of this plant was demonstrated against ear edema in mice produced by 12-*O*-tetradecanoylphorbol 13-acetate, and compounds **1**, **4**, and 3β-*O*-acetyloleanolic acid (**5**) were active when evaluated against cyclooxygenases-1 and -2.

The aerial parts of several species from the genus *Croton* are used in Mexico as anti-inflammatory and anticancer agents in folkloric medicine.[1] In continuation of our studies on Mexican *Croton* species,[2] we report herein an analysis of the chemical constituents of an *n*-hexane extract from the aerial parts of *Croton ciliatoglandulifer* Ortega (Euphorbiaceae; common name "sangre de drago"). Thus far, there have been no literature reports on the chemistry or biological activity of this species. The *n*-hexane, acetone, and methanol extracts of this plant were evaluated in an anti-inflammatory assay against ear edema in mice produced by 12-*O*-tetradecanoylphorbol 13-acetate (TPA, 0.10, 0.31, and 1.00 mg/ear),[3,4] and the *n*-hexane extract was active. All compounds included in the active fractions of this extract were tested for their ability to inhibit prostaglandin production by action of COX-1 and COX-2.[5]

Results and Discussion

In an anti-inflammatory activity test against ear edema in mice produced by TPA, an *n*-hexane extract of the aerial parts of *C. ciliatoglandulifer* showed activity (−8.59, −6.19, and 17.01% inhibition at a dose of 0.10, 0.31, and 1.0 mg/ear, respectively). In an assay-guided fractionation procedure, fractions F-2 (−13.4, 49.5, and 82.2% inhibition), F-4 (53.4, 58.3, and 79.0% inhibition), and F-5 (48.1, 38.9, and 48.16% inhibition) were also active at the same levels. Fractions F-2, F-4, and F-5 were purified and yielded the known natural products α- and β-amyrin,[6−8] 3β-*O*-acetyloleanolic acid (**5**),[9,10] *Z*,*Z*,*Z*,*Z*,*E*-hexaprenol,[11] a mixture of 3,7,3′-trimethylquercetin[12] and 3,7,4′-trimethylquercetin,[13] and the new phorbol derivatives 12-*O*-[(2*R*)-*N*,*N*-dimethyl-3-methylbutanoyl]-4-deoxyphorbol 13-acetate (**1**), 12-*O*-[(2*S*)-*N*,*N*-dimethyl-3-methylbutanoyl]-4-deoxyphorbol 13-acetate (**2**), 12-*O*-[3-methyl-2-butenoyl]-4-deoxyphorbol 13-acetate (**3**), and 12-*O*-[(2*R*)-*N*,*N*-dimethyl-3-methylbutanoyl]phorbol 13-acetate (**4**). The structure elucidation of compounds **1**−**4** is described herein.

Compound **1** was isolated as a dark brown oil with the ^{1}H and ^{13}C NMR data assigned as shown in Tables 1 and 2. On the basis of HRFABMS (M^+, *m*/*z* 517.3021), the molecular formula, $C_{29}H_{43}NO_7$, was deduced, representing nine degrees of unsaturation. Five of these were due to the presence of an α,β-unsaturated ketone, in accord with the absorptions at 1684 and 1455 cm^{-1} in the IR spectrum and the absorption maximum at 246 nm (ϵ 4847) in the UV spectrum, including signals for two ester carbonyl groups [δ_C 171.3 (s) and 173.4 (s)] and one trisubstituted double bond [δ_C 137.1 (s) and 125.8 (d)] in the ^{13}C NMR spectrum. The ^{13}C NMR and DEPT spectra of **1** showed 28 carbon resonances, corresponding to eight CH_3, two CH_2, ten CH, and eight quaternary carbons. An acetyl group was evident from signals at δ_H 2.01 (3H, s)/δ_C 21.1 (q) and 173.4 (s), a *N*,*N*-dimethyl group from the signal at δ_H 2.29 (6H, s)/δ_C 41.3, and a methylbutanoyl group from the signals at δ_C 171.3 (s), δ_H 2.73 (d)/δ_C 74.6, δ_H 1.97 (m)/δ_C 27.3, δ_H 0.95 (d)/δ_C 19.2, and δ_H 0.87 (d)/δ_C 19.8. The diterpenoid nature of **1** was deduced from the analysis of the 20 additional signals in the ^{13}C NMR spectrum, which were assigned as four CH_3, two CH_2, eight CH, and six quaternary carbons, consistent with a tetracyclic 4-deoxyphorbol skeleton.[14] The *cis* A/B ring junction was deduced by chemical shift values of H-1, H-5, H-7, and H-10, and C-1, C-2, C-3, C-4, C-5, C-10, and C-18 in the ^{1}H and ^{13}C NMR spectra.[15] The relative positions of the acetyl and methylbutanoyl



1 C-2′*R*, $R_1 = R_2 = H$

2 C-2′*S*, $R_1 = R_2 = H$

3 $\Delta^{2',3'}$, R = H

4 C-2′*R*, R_1 = OH, R_2 = H

1a C-2′*R*, R_1 = H, R_2 = COC_6H_5-*p*-Br

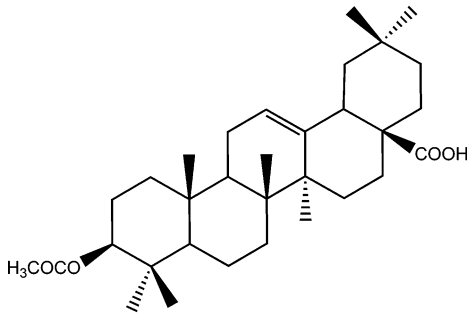


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Table 1. ^{1}H NMR Spectroscopic Data for Compounds **1**−**4** (400 MHz)[a]

position	**1**[b,c]	**2**[b,c]	**3**[b,c]	**4**[b,c]
1	6.99 bs	7.04 bs	7.05 bs	7.58 bs
4	2.71 m	2.80 ddd (6.4, 5.2, 3.2)	2.81 m	
5a	3.34 dd (15.6, 2.7)	3.39 dd (16.0, 2.8)	3.45 ddd (16.0, 2.8)	2.57 d (18.8)
5b	2.37 dd (15.6, 5.1)	2.50 dd (16.0, 5.2)	2.50 dd (16.0, 5.2)	2.48 d (18.8)
7	5.06 bs	5.12 bs	5.13 bs	5.69 d (6.0)
8	1.91 bs	2.02 bs	1.96 m	3.27 m
10	3.44 ddd (4.8, 4.4, 2.4)	3.51 ddd (6.4, 5.2, 2.0)	3.52 ddd (4.4, 4.4, 2.4)	3.25 m
11	1.67 dd (10.8, 6.6)	2.49 m	1.69 dq (10.4, 6.4)	2.17 m
12	5.49 d (10.8)	5.64 d (10.8)	5.51 d (10.4)	5.52 d (10.4)
14	0.78 d (4.8)	0.90 bs	0.79 d (5.2)	1.12 d (5.2)
16	1.15 s	1.21 s	1.21 s	1.25 s
17	1.08 s	1.13 s	1.18 s	1.17 s
18	1.05 d (6.6)	1.14 d (6.4)	1.09 d (6.4)	0.94 d (6.4)
19	1.71 bs	1.80 s	1.79 s	1.75 bs
20a	3.82 d (12.8)	4.02 d (12.8)	3.89 d (12.4)	4.05 d (13.2)
20b	3.92 d (12.8)	3.91 d (12.8)	4.02 d (12.4)	3.98 d (13.2)
2′	2.73 d (10.8)	2.87 m	5.71 dq (1.6, 1.2)	2.78 d (10.8)
3′	1.97 m	2.02 m		2.05 m
4′	0.95 d (6.6)	1.07 d (6.6)	2.21 d (1.2)	0.98 d (6.8)
5′	0.87 d (6.6)	0.98 d (6.6)	1.95 d (1.6)	0.89 d (6.8)
b	2.01 s	2.09 s	2.08 s	2.10 s
$N(CH_3)_2$	2.29 s	2.18 s		2.34 s
OH-9	5.06 bs	4.94 bs	5.21 bs	5.55 bs

[a] All assignments are based on 1D and 2D measurements (HSQC and HMBC). [b] δ/ppm multiplicity (J in Hz). [c] $CDCl_3$.

Table 2. ^{13}C NMR Spectroscopic Data for Compounds **1**−**4** (100 MHz)[a]

carbon	**1**[b−d]	**2**[b−d]	**3**[b−d]	**4**[b−d]
1	156.1 d	155.5 d	156.5 d	160.9 d
2	143.4 s	144.1 s	143.5 s	140.8 s
3	213.1 s	212.8 s	213.5 s	209.2 s
4	49.5 d	49.6 d	49.8 d	73.9 s
5	24.8 t	25.3 t	25.3 t	38.7 t
6	137.1 s	138.0 s	137.2 s	133.1 s
7	125.8 d	125.6 d	126.8 d	129.2 d
8	40.6 d	40.5 d	40.8 d	39.2 d
9	78.2 s	78.5 s	78.3 s	78.6 s
10	47.3 d	47.4 d	47.6 d	56.3 d
11	42.7 d	43.5 d	43.5 d	42.7 d
12	74.8 d	79.6 d	74.4 d	76.2 d
13	65.4 s	65.4 s	65.5 s	66.0 s
14	36.8 d	37.6 d	37.1 d	36.1 d
15	25.3 s	25.7 s	25.3 s	25.6 s
16	16.4 q	16.9 q	16.5 q	17.0 q
17	24.1 q	24.4 q	24.4 q	23.9 q
18	12.0 q	12.5 q	12.0 q	14.7 q
19	10.5 q	10.7 q	10.7 q	10.3 q
20	69.0 t	69.3 t	69.5 t	68.2 t
1′	171.3 s	168.0 s	166.4 s	171.7 s
2′	74.6 d	77.4 d	115.9 d	74.7 d
3′	27.3 d	28.4 d	158.1 s	27.5 d
4′	19.2 q	19.8 q	20.6 q	19.4 q
5′	19.8 q	22.6 q	27.7 d	19.8 q
a	173.4 s	173.1 s	173.8 s	173.9 s
b	21.1 q	21.4 q	21.3 q	21.3 q
$N(CH_3)_2$	41.3 q	42.5 q		41.4 q

[a] All assignments are based on 1D and 2D measurements (HSQC and HMBC). [b] δ/ppm multiplicity. [c] Multiplicities were determined by DEPT. [d] $CDCl_3$.

groups were established from the HMBC spectrum of **1** (Table S1), in which the signal at δ_H 5.49 (H-12) showed a cross-peak with the ester carbonyl signal at δ_C 171.3, while the signal at δ_H 2.01 (CH_3 acetyl) showed a cross-peak with the ester carbonyl signal at δ_C 173.4, establishing that the methylbutanoyl group is at C-12 and the acetyl group is at C-13. The *N*,*N*-dimethyl residue was assigned at C-2 of the methylbutanoyl group (C-2′ in **1**), in accord with the observation of cross-peaks between δ_H 2.73 (H-2′) and δ_H 1.97 (H-3′) and between H-3′ and δ_H 0.95 (H-4′) and δ_H 0.87 (H-5′) in the COSY spectrum (Table S1). Accordingly, compound **1** was proposed as 12-*O*-[(2*R*)-*N*,*N*-dimethyl-3-methylbutanoyl]-4-deoxyphorbol 13-acetate.

Compound **2** was obtained as a brown oil whose positive-ion HRFABMS (M^+, *m*/*z* 517.3040) data indicated the same molecular formula as that of **1** ($C_{29}H_{43}NO_7$). An α,*β*-unsaturated ketone was evident from the absorptions at 1676 and 1445 cm^{-1} in the IR spectrum and the absorption maximum at 256 nm (log ϵ 3.69) in the UV spectrum. This natural product showed practically the same ^{1}H and ^{13}C NMR data as **1** (Tables 1 and 2) with the following differences: an upfield shift for C-1′ ($\Delta\delta_C$ 168.0−171.3 = −3.3) and downfield shifts for C-2′ ($\Delta\delta_C$ 77.4−74.6 = 2.8) and H-2′ ($\Delta\delta_H$ 2.87−2.73 = 0.14), C-5′ ($\Delta\delta_C$ 22.6−19.8 = 2.8) and H-5′ ($\Delta\delta_H$ 0.98−0.87 = 0.11), and C-12 ($\Delta\delta_C$ 79.6−74.8 = 4.8) and H-12 ($\Delta\delta_H$ 5.64−5.49 = 0.15). These characteristics suggested that **2** is the C-2′ epimer of **1**.

The preferred conformations of **1** and **2** (Figure S1) derived from the MOPAC molecular modeling program were in agreement with the NMR data on the basis of the minor steric interaction between the *N*,*N*-dimethyl-3-methylbutanoyl group and the six-membered ring in compound **2** with respect to **1** ($\Delta\delta_{C-12}$ = 4.8, $\Delta\delta_{C-2'}$ = 2.9, and $\Delta\delta_{C-5'}$ = 2.8) and were supportive of compound **1** having an *R* and **2** an *S* configuration at C-2′. The C-2′ configuration was investigated through the NOESY spectroscopic data at 223 K for compound **1a**, the 20-*p*-bromobenzoyl derivative of **1**, in which H-2′ (δ_H 2.91) showed cross-peaks with the signals at δ_H 5.86 (H-12) and 1.02 (H-18), establishing that they have an α-orientation with respect to the phorbol ring system. Additionally, the isopropyl group (δ_H 1.07) showed a cross-peak with the signal at δ_H 5.86 (H-10), and the methyl groups of the *N*,*N*-dimethyl group (δ_H 2.35) showed a cross-peak with the signal of the methyl group of the 13-acetate at δ_H 1.55 (H−b), establishing that **1** is *R* and **2** is *S* at C-2′. Attempts to obtain a suitable crystalline derivative of **1** or **2** for X-ray diffraction structural confirmation were unsuccessful.

Compound **3** showed a molecular ion peak at *m*/*z* 473.2586 in the positive-ion HRFABMS, corresponding to the molecular formula $C_{27}H_{36}O_7$ and 10 degrees of unsaturation. This indicated that **3** has the same skeleton as **1** and **2** but without an *N*,*N*-dimethyl residue. Otherwise, this compound showed almost the same ^{1}H and ^{13}C NMR data as **1** and **2** (Tables 1 and 2). The additional unsaturation was assigned as a trisubstituted double bond, consistent with the observation of signals at δ_H 5.71 (dq, J = 1.6, 1.2 Hz)/δ_C 115.9 and 158.0 (s) in the ^{1}H and ^{13}C NMR spectra, and was located between C-2′−C-3′ due to the signals observed for C-4′ [δ_H 2.21 (d, J = 1.2 Hz)/δ_C 20.6] and C-5′ [δ_H 1.95 (d, J = 1.6 Hz)/δ_C 27.7]. In this manner, **3** was identified as 12-*O*-[3-methyl-2-

Table 3. IC_{50} Data of Compounds **1**−**5** as Inhibitors of COX-1 and COX-2[a,b]

compound	COX-1 (μM)	COX-2 (μM)
1	0.001	2.2
4	19.2	*c*
5	1.0	2.6
indomethacin	1.4	5.8

[a] Values are means of two determinations, and deviation from the mean is <5% ($n = 4-6$). [b] Compounds **2** and **3** were inactive in both test systems ($IC_{50} > 100$ μM). [c] Not analyzed since data did not show a linear regression.

butenoyl]-4-deoxyphorbol 13-acetate and its structure was in agreement with the cross-peaks observed in the COSY, HSQC, and HMBC spectra (Table S1).

Compound **4** was assigned as a phorbol derivative, in agreement with its ^{1}H and ^{13}C NMR spectroscopic data (Tables 1 and 2). This compound showed a $[M]^+$ peak at *m*/*z* 533.3017 in the HRFABMS, corresponding to a molecular formula of $C_{29}H_{43}NO_8$. In the ^{13}C NMR spectrum, **4** showed an additional signal at δ 73.9 compared to **1**, consistent with the presence of a hydroxyl group at C-4. This compound was assigned as 12-*O*-[(2*R*)-*N*,*N*-dimethyl-3-methylbutanoyl]phorbol 13-acetate.

Compounds **1**, **2**, and **4** include nitrogen in their molecules, and their occurrence in *Croton cilitoglandulifer* is interesting from a chemotaxonomic point of view because this is the first occasion that nitrogen-containing phorbol derivatives are reported for this genus. Nitrogen-containing phorbol derivatives are compounds of restricted distribution in the family Euphorbiaceae.[16]

The activity (IC_{50} values) of natural products **1**−**5** to inhibit the enzymes cyclooxygenases-1 (COX-1) and -2 (COX-2) was determined by enzymatic inhibitory assays. IC_{50} values for COX-1 and COX-2 and the concentration of prostaglandins in the evaluation of each natural product were obtained by means of extrapolation of data on standard curves. Compounds **2** and **3** were inactive in both assays ($IC_{50} > 100$ μM), while **1** and **5** were found to be more potent inhibitors to COX-1 than COX-2 (Table 3). In previous work, oleanolic acid inhibited the action of COX-2 at an IC_{50} value of 130 μM.[17] This current study has demonstrated that 3β-*O*-acetyloleanolic acid is 50 times more active than the parent compound, oleanolic acid, against COX-2.

Experimental Section

General Experimental Procedures. Optical rotations were measured on a Perkin-Elmer 241 MC polarimeter, and UV spectra were recorded on a Hewlett-Packard 8453 spectrometer using $CHCl_3$ as solvent. The IR spectra were obtained as films on a Bruker Vector 22 IR spectrometer. All NMR spectra were recorded on a Varian Unity 400 spectrometer at 400 MHz for ^{1}H NMR, ^{1}H−^{1}H COSY, HSQC, and HMBC and 100 MHz for ^{13}C NMR and ^{13}C DEPT, using $CDCl_3$ as solvent. Chemical shifts are reported in ppm (δ) relative to the TMS signal. FABMS and HRFABMS were recorded on a JEOL JMX-AX 505 HA mass spectrometer in a matrix of glycerol.

Plant Material. The aerial parts of *C. ciliatoglandulifer* (2−3 m shrub with red resin) were collected at Cañon de Lobos, Yautepec, Morelos, México, in August 2003 and were identified by Dra. Martha Martínez from Facultad de Ciencias Herbarium (FCME), UNAM, México (voucher specimen number 92581).

Extraction and Isolation. The air-dried parts of the plant material (4.38 kg) were powdered and extracted at room temperature with *n*-hexane (24 L × 3), acetone (24 L × 3), and methanol (24 L × 2) and concentrated to dryness in vacuo to obtain 180, 137, and 108 g of residue, respectively. These extracts were analyzed in an anti-inflammatory assay against ear edema in mice produced by TPA, and the *n*-hexane extract was active (−8.59, −6.19, and 17.01% inhibition at a dose of 0.10, 0.31, and 1.0 mg/ear, respectively), while the acetone and methanol extracts showed nonlinear responses at the same concentrations. The *n*-hexane extract was purified by open column chromatography with an *n*-hexane−acetone gradient, collecting fractions of 500 mL each. The composition of the obtained fractions was monitored by means of TLC, and the compounds were visualized under UV light and by spraying with a 1% solution of $CeSO_4 \cdot NH_3$ in 2 N H_2SO_4. On the basis of TLC, the fractions were pooled into eight groups: F-1 (10.15 g, *n*-hexane, 100%), F-2 (7.10 g, *n*-hexane−acetone, 95:5), F-3 (5.73 g, *n*-hexane−acetone, 9:1), F-4 (13.09 g, *n*-hexane−acetone, 8:2), F-5 (3.0 g, *n*-hexane−acetone, 7:3), F-6 (4.15 g, *n*-hexane−acetone, 6:4), F-7 (5.52 g, *n*-hexane−acetone, 5:5), and F-8 (8.12 g, acetone, 100%). Fractions F-2, F-4, and F-5 showed inhibition of ear edema in mice. Each fraction was further separated using column chromatography over silica gel 60 and a gradient of *n*-hexane−acetone as eluent in each case. Fraction F-2 yielded *Z*,*Z*,*Z*,*Z*,*E*-hexaprenol (358 mg, 0.20%), α- and β-amyrin (153 mg, 0.09%), and 3β-*O*-acetyloleanolic acid (**5**, 89 mg, 0.05%); fraction F-4 yielded a mixture of 3,7,3′-trimethylquercetin and 3,7,4′-trimethylquercetin (473 mg, 0.26%), and 12-*O*-[(2*R*)-*N*,*N*-dimethyl-3-methylbutanoyl]-4-deoxyphorbol 13-acetate (**1**, 236 mg, 0.13%). Finally, fraction F-5 yielded 12-*O*-[(2*S*)-*N*,*N*-dimethyl-3-methylbutanoyl]-4-deoxyphorbol 13-acetate (**2**, 4.7 mg, 0.003%), 12-*O*-[3-methyl-2-butenoyl]-4-deoxyphorbol 13-acetate (**3**, 4.3 mg, 0.002%), and 12-*O*-[(2*R*)-*N*,*N*-dimethyl-3-methylbutanoyl]phorbol 13-acetate (**4**, 17.2 mg, 0.009%).

12-*O*-[(2*R*)-*N*,*N*-Dimethyl-3-methylbutanoyl]-4-deoxyphorbol 13-acetate (1): dark brown oil; $[\alpha]^{25}_D$ −5.2 (*c* 0.94, $CHCl_3$); UV ($CHCl_3$) λ_{max} (log ϵ) 246 (3.69), 431 (2.54) nm; IR (KBr) ν_{max} 3417, 2927, 1724, 1684, 1455, 1275, 1248, 1151, 1118, 1051 cm^{-1}; ^{1}H NMR ($CDCl_3$, 400 MHz) and ^{13}C NMR ($CDCl_3$, 100 MHz), see Tables 1 and 2; (+)-FABMS *m*/*z* 518 $[M + H]^+$ (30), 500 $[M + H - H_2O]^+$ (13), 474 $[M + H - N(CH_3)_2]^+$ (2), 473 $[M - N(CH_3)_2]^+$ (3), 355 (14), 307 (33), 154 (97), 136 (82), 100 (100); HRFABMS *m*/*z* 517.3021 $[M]^+$ (calcd for $C_{29}H_{43}NO_7$, 517.3028).

12-*O*-[(2*S*)-*N*,*N*-Dimethyl-3-methylbutanoyl]-4-deoxyphorbol 13-acetate (2): brown oil; $[\alpha]^{25}_D$ −3.0 (*c* 0.23, $CHCl_3$); UV ($CHCl_3$) λ_{max} (log ϵ) 256 (3.69), 249 (3.46), 303 (1.79) nm; IR ($CHCl_3$) ν_{max} 3403, 2927, 1722, 1676, 1462, 1445, 1375, 1326, 1259, 1152 cm^{-1}; ^{1}H NMR ($CDCl_3$, 400 MHz) and ^{13}C NMR ($CDCl_3$, 100 MHz), see Tables 1 and 2; (+)-FABMS *m*/*z* 518 $[M + H]^+$ (12), 500 $[M + H - H_2O]^+$ (2), 473 $[M - N(CH_3)_2]^+$ (2), 307 (57), 154 (100), 137 (100); HRFABMS *m*/*z* 517.3040 $[M]^+$ (calcd for $C_{29}H_{43}NO_7$, 517.3028).

12-*O*-[3-Methyl-2-butenoyl]-4-deoxyphorbol 13-acetate (3): brown oil; $[\alpha]^{25}_D$ −6.0 (*c* 0.21, $CHCl_3$); UV ($CHCl_3$) λ_{max} (log ϵ) 248 (3.51), 414 (1.98) nm; IR ($CHCl_3$) ν_{max} 3414, 2924, 1715, 1644, 1459, 1376, 1251, 1089 cm^{-1}; ^{1}H NMR ($CDCl_3$, 400 MHz) and ^{13}C NMR ($CDCl_3$, 100 MHz), see Tables 1 and 2; (+)-FABMS *m*/*z* 473 $[M + H]^+$ (4), 307 (76), 154 (100), 137 (100); HRFABMS *m*/*z* 473.2586 $[M + H]^+$ (calcd for $C_{27}H_{37}O_7$, 473.2529).

12-*O*-[(2*R*)-*N*,*N*-Dimethyl-3-methylbutanoyl]phorbol 13-acetate (4): brown oil; $[\alpha]^{25}_D$ +36.7 (*c* 0.86, $CHCl_3$); UV ($CHCl_3$) λ_{max} (log ϵ) 249 (3.46), 303 (1.79) nm; IR ($CHCl_3$) ν_{max} 3403, 2927, 1722, 1462, 1375, 1326, 1259, 1152 cm^{-1}; ^{1}H NMR ($CDCl_3$, 400 MHz) and ^{13}C NMR ($CDCl_3$, 100 MHz), see Tables 1 and 2; (+)-FABMS *m*/*z* 534 $[M + H]^+$ (8), 389 (5), 311 (10), 154 (17), 146 (31), 100 (100); HRFABMS *m*/*z* 533.3017 $[M]^+$ (calcd for $C_{29}H_{43}NO_8$, 533.2977).

12-*O*-[(2*R*)-*N*,*N*-Dimethyl-3-methylbutanoyl]-20-*p*-bromobenzoyl-4-deoxyphorbol 13-acetate (1a). To a well-stirred solution of the alcohol **1** (244 mg, 0.47 mmol) in CH_2Cl_2 (5 mL) were added DMAP (catalytic amount), *p*-bromobenzoyl chloride (1.5 equiv, 0.70 mmol, 155 mg), and Et_3N (1.5 equiv, 0.70 mmol, 70.7 mg, 0.097 mL). The reaction mixture was stirred at room temperature for 2.5 h. Brine (5 mL) was added to the reaction mixture, and the organic phase was separated. The aqueous phase was extracted with CH_2Cl_2 (3 × 10 mL). The combined organic layers were dried over Na_2SO_4, filtered, and concentrated. The residue was purified by flash column chromatography using a mixture of dichloromethane−acetone (95:5) as eluent: yellow powder (106.7 mg); ^{1}H NMR ($CDCl_3$, 400 MHz, 223 K) δ 8.01 (2H, d, $J = 8.8$ Hz, H-2″,6″), 7.19 (2H, d, $J = 8.8$ Hz, H-3″,5″), 6.38 (1H, bs, H-1), 5.83 (1H, d, $J = 10.8$ Hz, H-12), 5.30 (1H, s, OH-9), 5.13 (1H, bs, H-7), 4.75 (1H, d, $J = 12.8$ Hz, H-20a), 4.61 (1H, d, $J = 12.8$ Hz, H-20b), 3.58 (1H, dd, $J = 15.2$, 3.2 Hz, H-5a), 3.20 (1H, ddd, $J = 6.8$, 2.4, 2.0 Hz, H-10), 2.83 (1H, d, $J = 10.8$ Hz, H-2′), 2.71 (1H, dd, $J = 15.2$, 4.4 Hz, H-5b), 2.47 (1H, dt, $J = 6.8$, 4.4 Hz, H-4), 2.36 (6H, bs, $N(CH_3)_2$), 2.05 (1H, m, H-3′), 1.94 (1H, bs, H-8), 1.64, (6H, bs, H-19, H−b), 1.59 (1H, m, H-11), 1.10 (3H, d, $J = 6.0$ Hz, H-18), 1.04 (1H, d, $J = 6.4$ Hz, H-4′), 1.03 (3H, s, H-16), 0.98 (1H, d, $J = 6.4$ Hz, H-5′), 0.95 (3H, s, H-17), 0.74 (1H, d, $J = 4.8$ Hz, H-14); ^{13}C NMR ($CDCl_3$, 100 MHz) δ 209.2 (C, C-3), 173.0 (C, C−a), 170.8 (C, C-1′), 165.4 (C, C-a″), 154.0 (CH, C-1), 143.4 (C, C-2), 134.0 (C,

C-1″), 133.9 (C, C-6), 131.8 (CH, C-2″,6″), 131.7 (CH, C-3″,5″), 130.1 (C, C-4″), 129.4 (CH, C-7), 78.2 (C, C-9), 74.8 (CH, C-2′), 74.6 (CH, C-12), 71.8 (CH_2, C-20), 65.5 (C, C-13), 49.1 (CH, C-4), 47.2 (CH, C-10), 42.7 (CH, C-11), 41.4 (CH_3, $N(CH_3)_2$), 41.1 (CH, C-8), 37.0 (CH, C-14), 27.9 (CH, C-3′), 26.8 (C, C-15), 26.8 (CH_2, C-5), 23.9 (CH_3, C-17), 21.0 (CH_3, C-b), 19.9 (CH_3, C-5′), 19.3 (CH_3, C-4′), 16.2 (CH_3, C-16), 12.1 (CH_3, C-18), 10.5 (CH_3, C-19); (+)-FABMS *m*/*z* 702 $[M + 2]^+$ (89), 700 $[M]^+$ (91), 500 (59), 458 (21), 355 (73), 313 (100), 295 (85), 267 (22); HRFABMS *m*/*z* 700.2447 $[M + H]^+$ (calcd for $C_{36}H_{47}O_8N^{79}Br$, 700.2472); 702.2498 $[M + H + 2]^+$ (calcd for $C_{36}H_{47}O_8N^{81}Br$, 702.2452).

Chemicals and Enzymes. TPA (12-*O*-tetradecanoylphorbol 13-acetate), sedalphorte (sodium pentobarbital), indomethacin, dimethyl sulfoxide, and methanol were purchased from Sigma Chemical Co. (Milwaukee, WI), and prostaglandin screening EIA (enzyme immunoassay) antiserum, prostaglandin screening AChE (acetylcholinesterase) tracer, prostaglandin screening EIA standard, EIA buffer (10×), wash buffer (400×), Tween 20, mouse anti-rabbit IgG coated plate, Ellman's reagent, reaction buffer (10×), COX-1 (ovine), COX-2 (human recombinant), heme, arachidonic acid (substrate), potassium hydroxide, hydrochloric acid, and stannous chloride were purchased from Cayman Chemical Co. (Ann Arbor, MI).

Mouse Ear-Inflammatory Assay. The anti-inflammatory activity of extracts and fractions was evaluated against ear edema in mice produced by TPA, according to a previously described procedure.[3,4]

Cyclooxygenase Inhibition Studies. All compounds isolated in the in vivo active anti-inflammatory fractions from the *n*-hexane extract were tested for their ability to inhibit prostaglandin production by action of COX-1 and COX-2, using COX-1 (ovine) and COX-2 (human recombinant) inhibitor screening kits according to the manufacturer's instructions. Stock solutions of test compounds (inhibitor) were prepared to concentrations of 10, 100, and 1000 μM in DMSO. To a series of supplied reaction buffer solutions (950 μL, 0.1 M Tris-HCl (pH 8.0) containing 5 mM EDTA and 2 mM phenol) with COX-2 (10 μL) enzyme in the presence of heme (10 μL) was added 20 μL of test compounds. These solutions were incubated for a period of 10 min at 37 °C, after which 10 μL of arachidonic acid (100 μM) was added, and the reaction was stopped by the addition of 50 μL of 1 M HCl after 2 min. Prostaglandin $F_{2\alpha}$ produced by reduction from PGH_2 with stannous chloride (100 μL was added and incubated for 5 min at room temperature) was quantified via enzyme immunoassay (EIA) using a mouse anti-rabbit IgG monoclonal antibody (previously attached to the well) that binds to all the prostaglandin compounds. The plate was washed to remove any unbound reagents, and then Ellman's reagent (which contains the substrate to AChE) was added to the well. The product of this enzymatic reaction showed a yellow color and absorbed strongly at 405 nm. The intensity of this color, determined spectrophotometrically, is proportional to the amount of PG tracer bound to the well, which was inversely proportional to the amount of free PG present in the well during the incubation. The concentration of the test compound causing 50% inhibition (IC_{50}, μM) was calculated from the concentration−inhibition response curve (duplicate determinations) according to the manufacturer's instructions.

Statistical Analysis. The ANOVA test was used for the TPA-induced ear edema test ($p < 0.05$ was considered to be significant). Analysis of the regression line for the COX-1 and COX-2 assay was used to calculate IC_{50} values.

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Supporting Information Available: Preferred conformations of **1** and **2** determined by molecular modeling, and table of 2D NMR data for compounds **1**, **1a**, **2**, and **3**. This information is available free of charge via the Internet at http://pubs.acs.org.

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